

Modified nucleotides reveal the indirect role of the central base pairs in stabilizing the *lac* repressor–operator complex

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Received February 3, 1995; Revised and Accepted March 13, 1995

ABSTRACT

Guanine residues in the *lac* operator were replaced by 2-aminopurine or purine analogues, pairing the modified nucleotides with C. The observed equilibrium dissociation constants for *lac* repressor binding to substituted operators were measured in 10 mM Tris, 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTE, pH 7.6 at 25°C. These measurements revealed five positions that destabilized the complex when substituted with either analogue. Two positions, which are related by a 2-fold symmetry, are in the major groove of the operator thought to directly interact with the protein. Three sites were in the central region of the operator. A purine analogue at a sixth site perturbed the local DNA structure and destabilized the complex. Alkylation interference experiments of the 2-aminopurine substituted operators demonstrated that, of the five affected, two substitutions displayed altered phosphate interference patterns at the phosphate adjacent to the substituted base. For these operators, complex formation was measured in different concentrations of KCl to assess the contribution of counterion release to the bimolecular process. The results indicated that both complexes were similar to wild-type, although minor changes were observed. The K_{obs} of the complex was then measured when 2-aminopurine or purine analogues were paired with uracil nucleotide, a base pair that serves to stabilize the DNA. The introduction of the new base pairs revealed two effects on the bimolecular interaction. For those operator sites that are thought to perturb the interaction directly, the affinity of the complex was weakened to levels observed for the singly-substituted operators. In contrast, the nucleotides of 2-aminopurine paired with uracil positioned in the central region of the operator served to enhance the stability of the complex. The purine–uracil base pair substitution on the other hand had a significant destabilizing effect on the interaction. We propose that the central base pairs modulate binding of the complex by altering the intrinsic properties of the DNA. Two

specific attributes are required to achieve the lowest free energy of interaction. The DNA must have two interstrand hydrogen bonds to stabilize the duplex and it must have properties associated with directional bending or unwinding. This analysis does not rule out contributions by direct interactions between the protein and the central region of the operator but underscores how indirect effects play a major role in complex formation in this system.

INTRODUCTION

Understanding the role DNA plays in facilitating the association of DNA-binding proteins is necessary for understanding how sequence specificity is accomplished by this class of proteins. Direct interactions generated by hydrogen bonding or hydrophobic interactions are clearly required for complex formation. However, consideration must be given to how DNA structure is altered when a protein binds and what intrinsic properties of the DNA are required in order to achieve the lowest free energy of binding.

Recent crystallographic work has provided structural insights into the role of DNA in complex formation, summarized in a number of reviews (1–3). Solution studies using natural base pair mutations have been used to gauge contributions by the intrinsic properties of DNA to complex formation and complement recent crystallographic analyses. Two examples of combined solid state and solution properties are the CAP protein, which has two protein-induced 45° bends in the DNA (4), and the 434 repressor which requires a specific sequence to be present in the central region of the recognition sequence in order to achieve the lowest free energy of binding (5). Most recently the PurR protein, a DNA binding protein of the LacI family, was shown to have a 45° kink in the central region of the DNA recognition site (6).

Modified nucleotides have contributed significantly to defining protein–DNA interactions. Singly substituted analogues introduced into the DNA are structurally isosteric and have been used by a number of investigators for identifying essential substituent groups necessary for a given protein–DNA interaction (7–14). The use of ‘anomalous’ base pairs, namely the pairing of two complementary modified nucleotides, was recently used by the

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McLaughlin and co-workers (15). Although this may result in the alteration of multiple chemical groups in the DNA, these complementary analogues when used in conjunction with singly substituted analogues allow for a more careful analysis of DNA-binding proteins contact sites.

In this work we have extended the use of analogue base pair substitutions by focusing on possible effects that these analogues have on the intrinsic properties of the DNA. By considering possible alterations to the physical properties of the DNA and correlating them with the stability of the protein–DNA complex, we were able to further characterize contributions made by the DNA to the association process which are unrelated to contact sites. These studies, coupled with available structural information for the DNA and the complex, revealed direct and indirect roles of the DNA in the recognition process.

Specifically, a combination of both singly and doubly substituted operators was used to study the *lac* repressor–operator interaction. We have shown that for sites in the operator that are thought to interact directly through intermolecular hydrogen bonding (16,17), the replacement of G with any modified nucleotide resulted in an expected perturbation of the stability of the complex attributed to the positioning of sterically incompatible groups at the DNA–protein interface. In contrast, the insertion of analogues in the central region of the operator modulated the complex's stability, depending on the presence of single or double substitutions. For singly substituted operators containing either 2-aminopurine or purine nucleotides, K_{obs} indicated that the complex was destabilized. With the introduction of the 2-aminopurine–uracil nucleotide base pair, the thermodynamic stability of the complex was enhanced, and in two cases the complex had a more favorable free energy of binding than that of the wild-type complex. This was not true for purine–uracil base pairs whose insertion into the operator DNA served to destabilize the complex. Based on the available thermodynamic data for these analogue nucleotide base pairs, we concluded that both the stability generated by two interstrand hydrogen bonds and directional deformation of the DNA are needed in the central region of the operator for complex formation.

MATERIALS AND METHODS

2-Aminopurine and purine-9- β -D-2'-deoxyribofuranoside nucleoside analogue phosphoramidites

The protected phosphoramidite of 2-aminopurine-9- β -D-2'-deoxyribofuranoside was synthesized as previously described (18). The protected phosphoramidite of purine and uracil nucleotides were purchased from Glen Research.

Synthesis and purification of oligonucleotides

Chemical synthesis was carried out on an Applied Biosystems Inc. model 381A DNA synthesizer. Removal of protecting groups was achieved by heating the material to 50°C for 6 h in a 30% ammonia solution. Subsequently, the solvent was removed by evaporation and the DNA was purified by denaturing gel electrophoresis (20% polyacrylamide–7M urea). Purity of the oligonucleotide was assessed by phosphorylating the DNA with T₄ polynucleotide kinase and [γ -³²P]ATP, and analyzed by denaturing gel electrophoresis (45 V/cm). The wild-type sequence was confirmed by the chemical sequencing and each

analogue operator was subjected to purine sequencing reactions (19).

DNA duplex preparation

DNA concentration was determined by UV absorbance at 260 nm and corrected for nearest neighbor effects (20). Radiolabeled duplex DNA was prepared by taking single-stranded DNA (4 pmol) and incubating it with T₄ polynucleotide kinase and [γ -³²P]ATP at 37°C for 30 min, followed by ethanol precipitation. Unlabeled complementary strand was added at a 20% excess to the labeled DNA to a final volume 40 μ l of 10 mM Tris, 150 mM KCl, 0.1 mM EDTA, at pH 7.6. The solution was heated to 90°C, cooled slowly and stored at 4°C overnight. Excess DNA was required for quantitative annealing of the labeled strand. The duplex DNA was assessed on 10% PAGE and was found to be >99% in duplex form. The concentration of operator was calculated from the specific activity of the radiolabeled strand.

Protein activity

The activity of *lac* repressor protein (a gift of Mitch Lewis, Helen Pace and Ponzy Lu from the University of Pennsylvania) was measured at a concentration of protein and DNA well above the equilibrium dissociation constant. Labeled wild-type duplex DNA at a concentration of 1×10^{-8} M was incubated with varying dilutions of repressor protein whose concentration was previously determined by UV absorbance (2.1×10^{-4} M) in binding buffer and at 25°C. All samples were incubated for 30 min and then filtered through nitrocellulose filters, dried and counted. Protein concentrations tested were done in duplicate. The total counts retained versus the dilution were plotted, and filter efficiency was determined by comparing total amount of DNA added to each sample and the quantity retained after filtration at saturation. The activity of the protein was extracted from the linear portion of this graph, assuming a ratio of one operator to tetrameric repressor protein. This latter assumption is based on an upper limit set for the dimer to tetramer equilibrium constant (21–24). The activity of *lac* repressor was between 80 and 100%.

Competition equilibrium dissociation assays

The equilibrium binding constant was measured using the competition method (25,26). The wild-type DNA (2 pmol) was labeled (see above) and radioactive ATP was removed by passing the reaction mixture through a Sephadex G-50 column equilibrated with 10 mM Tris, pH 7.6, 150 mM KCl and 0.1 mM EDTA. Assays were done in a final volume of 200 μ l of a solution containing 10 mM Tris, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 1 mM DTE and 50 μ g/ml BSA. After 30 min at ambient temperature, the mixture was filtered on pre-soaked nitrocellulose filters, washed once with 500 μ l of buffer (10 mM Tris, pH 7.6, 150 mM KCl and 0.1 mM EDTA) and dried. Typically, wild-type operator DNA (1.0×10^{-11} M) and repressor (5.0×10^{-11} M) were incubated with varying concentrations of competitor DNA. Duplicate samples of protein concentrations were tested and each assay was repeated at least twice. Background was determined by the addition of IPTG (1 mM final concentration) to the protein–DNA complex. The results were fitted to a binding curve (25,26) using non-linear regression analysis with SIGMA-PLOT software for the equation shown below:

$$RO_{cpm} = \frac{O_m}{2O_t} \left[K_{RO} + \frac{K_{RO}}{K_{RC}} C_t + R_t + O_t \sqrt{\left(K_{RO} + \frac{K_{RO}}{K_{RC}} C_t + R_t + O_t \right)^2 - 4O_t R_t} \right]$$

Where R_t , K_{RO} , K_{RC} , C_t and O_t are the total protein, equilibrium dissociation constant for wild-type operator, equilibrium dissociation constant for competitor, total competitor DNA, and total labeled operator, respectively. The unit of RO from the assay is c.p.m. (counts/min).

The competition curve related to the amount of labeled complex formed as a function of increasing concentrations of competitor DNA was fitted to the best value for the dissociation constant K_{RC} . The value O_m (the total available operator for binding) was derived from the amount of complex available at saturating concentrations of protein. The concentrations of wild-type repressor and operator were chosen that set the initial value of RO (RO_o) at 50–80% of the total counts (O_m). The starting concentration RO_o can be varied without affecting the derived equilibrium dissociation constant. Duplicate samples were performed for each concentration and each operator sequence was tested at least twice. Using the wild-type operator as competitor DNA, the observed equilibrium dissociation constant was measured and was in good agreement with values obtained for the standard equilibrium assay at 1.2×10^{-11} M (14,27). The competition equation shown above is correct when $C_t \gg RC$ (RC is the concentration of the competitor–repressor complex) and can not be used when the modified operator binds tighter than the wild-type operator. For these cases, an alternate form of the equation was used that makes no *a priori* assumptions. These results were confirmed by a standard equilibrium assay which gave identical values in both assays (data not shown).

For all these measurements, the repressor protein was assumed to be in the dimer form. Although controversy surrounds the exact value of the dimer to tetramer equilibrium constant (22–24), we have shown using polyacrylamide gel electrophoresis that a single population of repressor is bound to DNA and that it co-migrates with a mutant dimer form of repressor bound to the identically sized DNA (28).

Measurement of the salt dependency of the complex

The equilibrium binding of proteins to nucleic acids is affected by the concentration of monovalent and divalent salts. Record and co-workers have derived specific equations which relate the formation of a protein–DNA complex with counterion release (29,30). [Recently, the tetrameric form of repressor was shown to bind a single chloride ion and to stabilize the looped structure of DNA and tetrameric repressor (21). However, the chloride ion appears not to affect the intrinsic binding constant.] In the absence of specific anion effects and at low concentrations of salts, the observable dissociation equilibrium constant is related to the concentration of KCl by the following equation:

$$\frac{d \ln K_{obs}}{d \ln [KCl]} = 0.88Z$$

where 0.88Z is the net number of counterions released upon association of the complex. The equilibrium constants of *lac* repressor binding to the wild-type or modified operators were measured at KCl concentrations ranging between 125–200 mM using the competition filter-binding method described above.

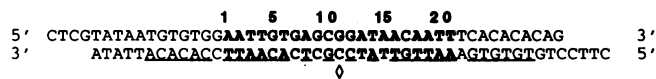


Figure 1. Depicted is the synthetic 43 bp sequence used for competition equilibrium dissociation assays. This fragment is derived from the *lacUV5* operon. The operator sequence is shown in bold and regions of symmetry are underlined.

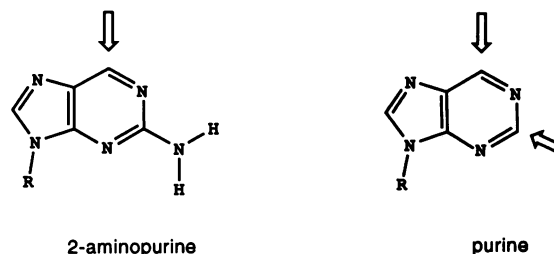


Figure 2. Chemical structure of the nucleobase analogues of 2-aminopurine and purine. Arrows indicate sites of modification and R refers to the deoxyribose.

RESULTS

Wild-type and modified operators

Operator sequences with or without the modified nucleotide were constructed from two complementary synthetic strands to form a 43 bp duplex with a four base overhang at both ends. The sequence of DNA, the *lac* operon called UV-5, is shown in Figure 1 along with the numbering scheme referenced in this work. In the first set of experiments, seven different substituted operator sequences were synthesized with a single G residue replaced by either 2-aminopurine or purine nucleotide whose chemical structures are shown in Figure 2. This allowed seven operators to be formed, each having a single substitution at positions 5, 7, 9, 11 or 12 in the top strand or at positions 10 or 17 in the bottom strand. In each case the modified nucleotide was paired with C. Subsequently, a second set of experiments were done using 2-aminopurine (2AP) or purine (P) paired with uracil (U) nucleotides.

Equilibrium dissociation constants of complexes containing 2AP–C base pairs

The effect of inserting 2-aminopurine (2AP) into the *lac* operator was measured using the competition filter binding assay (Fig. 4A; Table 1). Two symmetrically related modified operators, one at position 5 and one at position 17 significantly destabilized the complex. A larger energetic penalty was associated with the O^{2AP5} (*lac* operator substituted with 2AP at position 5) than with the substitution O^{2AP17} [$\Delta\Delta G^\circ(O^{2AP5}) = 3.3$ kcal/mol versus $\Delta\Delta G^\circ(O^{2AP17}) = 2.3$ kcal/mol]. This was consistent with previous work in which modifications made in the left half of the operator sequence resulted in larger free energy changes than those made in the right, although both are symmetrically related (9). Three sites located in the central region of the operator when substituted with 2AP analogue also resulted in an increase in positive free energy. These changes were at positions 9 and 11 on

Table 1. Equilibrium dissociation constants for the wild-type and the modified operator sequences derived from the competition assay. The pairing of the modified nucleotide is indicated below

	1	5	10	15	20			
5'	AATTGTGAGCGGATAACAATT					3'		
3'	TTAACACTCGCCTATTGTTAA					5'		
2AP·C		P·C		2AP·U		P·U		
	K_{obs}^a 10 ⁻¹¹ M	$\Delta\Delta G^{ob}$ kcal/mol	K_{obs} (10 ⁻¹¹ M)	$\Delta\Delta G^o$ kcal/mol	K_{obs} (10 ⁻¹¹ M)	$\Delta\Delta G^o$ kcal/mol	K_{obs} (10 ⁻¹¹ M)	$\Delta\Delta G^o$ kcal/mol
wild-type	1.3 ± 0.3	—	—	—	—	—	—	—
5	312 ± 62	3.3 ± 0.3	1770 ± 410	4.3 ± 0.3	148 ± 26	2.8 ± 0.2	230 ± 30	2.8 ± 0.3
7	2.5 ± 0.5	nc ^c	8.1 ± 2.2	1.1 ± 0.3	54 ± 14	2.2 ± 0.2	38 ± 7	1.7 ± 0.3
9	8.4 ± 0.7	1.2 ± 0.1	9.3 ± 1.8	1.2 ± 0.2	2.7 ± 0.9	0.4 ± 0.2	6.9 ± 0.6	0.7 ± 0.3
10	40 ± 11	2.1 ± 0.2	25 ± 6	1.8 ± 0.2	0.1 ± 0.02	-1.6 ± 0.3	17 ± 4	1.2 ± 0.4
11	30 ± 4	1.9 ± 0.2	86 ± 18	2.5 ± 0.3	0.38 ± 0.05	-0.8 ± 0.2	43 ± 4	1.8 ± 0.2
12	1.3 ± 0.2	nc	1.0 ± 0.1	nc	1.5 ± 0.2	nc	0.8 ± 0.2	-0.6 ± 0.3
17	59 ± 10	2.3 ± 0.2	62 ± 11	2.3 ± 0.2	22 ± 3.5	1.6 ± 0.2	52 ± 7	1.9 ± 0.3

^aBinding was measured in 10 mM Tris, 150 mM KCl, at pH 7.6, and 25°C. Constants and their S.D. are tabulated above and are the average of at least three independent determinations. The equilibrium constants determined assume one binding site per DNA molecule.

^b $\Delta\Delta G^o = RT \ln[K_{obs}(\text{substitution})/K_{obs}(\text{wild-type})]$. The observable binding constant for wild-type used to calculate the change in free energy of binding varies from assay to assay. Consequently, the above wild-type value when used to calculate $\Delta\Delta G^o$ gives rise to a small apparent error.

^cNo change

the top strand [$\Delta\Delta G^o(O^{2AP9}) = 1.2$ kcal/mol and $\Delta\Delta G^o(O^{2AP11}) = 1.9$ kcal/mol], respectively, and at position 10 on the bottom strand ($\Delta\Delta G^o(O^{2AP10}) = 2.1$ kcal/mol).

Equilibrium dissociation constants for complexes containing 2AP·U base pairs

To further assess the role of duplex stability on the bimolecular process, we measured the affinity of the repressor protein for operator when the *lac* operator was substituted with 2-aminopurine-uracil base pairs (2AP·U) instead of the 2-aminopurine-cytosine. Thermal denaturation experiments have shown that duplexes containing 2AP paired with thymine are more stable than duplexes containing 2-aminopurine paired with cytosine (31–33). The use of the uracil analogue was dictated by the need to mimic the structure of cytosine which does not contain a 5-methyl group in the major groove.

The new base pair 2AP·U placed at either position 5 or 17 showed small but favorable improvements in the free energy of interaction when compared to the 2AP·C base pair, although significant destabilizing effects were still evident compared to the wild-type operator (see Fig. 4B, Table 1). The position 5 substitution had a $\Delta\Delta G^o = 2.8$ kcal/mol, an increased stability of -0.5 kcal/mol compared to the 2AP·C base pair, while the position 17 substituted operator showed a similar incremental change of approximately -0.7 kcal/mol (Table 1). These results are consistent with perturbations to direct interactions of the protein–DNA complex and resulted from the positioning of sterically incompatible groups at the protein–DNA interface (see Discussion).

In contrast, positioning the 2AP·U base pairs in the central base pairs at position 9, 10 and 11 had large favorable changes in affinity, improving the binding near to or better than wild-type levels (Fig. 4B; Table 1). The 2AP·U base pair at position 10 was the most striking example of the three sites, having a $\Delta\Delta G^o = -1.6$

kcal/mol compared with wild-type. A comparison of free energy values of 2AP·U with 2AP·C showed an overall favorable increase in stability of -3.7 kcal/mol. Similar improvements were observed for the position 11 substitution which had an overall change of -2.7 kcal/mol. While the third site at position 9 of the central region was also favorably affected, the magnitude of change was smaller, with an overall negative free energy change of -0.8 kcal/mol.

The only substituted position which resulted in a positive free energy change with the new base pair scheme was the operator substituted at position 7 (Fig. 4B; Table 1). Since the 2AP·C base pair did not alter the stability of the complex, we concluded that the carbonyl of uracil served to further destabilize the interaction [$\Delta\Delta G^o = 2.2$ kcal/mol].

Equilibrium dissociation constants for complexes containing P·C base pairs

Similar experiments to the ones described above were done with the purine analogue (P). The same five sites perturbed the interaction, although not necessarily to the same extent as 2AP, as well as an additional substitution at position 7 (Fig. 4C; Table 1). Comparing the P and 2AP effects at individual sites, we found that O^P5 operator had an additional 1 kcal/mol, while the same analogue at position 17, O^P17 , was identical to the 2AP substitution. On the other hand, the insertion of the purine analogue at either position 9 or 10 had a positive free energy change that was similar in magnitude to the 2-aminopurine at these sites. Only O^P11 destabilized the interaction by an additional 0.6 kcal/mol when compared to the 2AP. The position 7 substitution which was unaffected by the 2AP substitution had a free energy change of ~1 kcal/mol (Table 1).

Equilibrium dissociation constants for complexes containing P·U base pairs

We measured the K_{obs} for purine residue paired with the uracil (P·U) at the same seven guanine sites (see Fig. 4D; Table 1), allowing us to compare the effect of the P·C and the 2AP·U base pairs with that of the P·U. The 2AP·U base pair, which has two hydrogen bonds, is presumed to be more stable than the P·U base pair since it can only accommodate a single inter-strand hydrogen bonding site and the P·U base pair is more stable than P·C base pair (see Fig. 3).

The P·U base pair substituted operators had a similar effect on the complex stability to that of 2AP·U base pair substitutions at positions 5, 7, 9 and 17 of the *lac* operator. For example, the differences observed for substitutions at positions 9 or 17 were 0.3 kcal/mol. Similar results were obtained for substitutions at positions 7 and 12 in which the free energy changes were relatively small, ~0.5 kcal/mol, enhancing the stability of the complex in comparison to the 2AP·U modifications.

Larger energy fluctuations in the free energy of binding were observed at positions 10 and 11 of the operator (see Fig. 4; Table 1). The purine analogue paired with uracil instead of 2-aminopurine analogue base resulted in a $\Delta\Delta G^o = 1.2$ kcal/mol at position 10 and a $\Delta\Delta G^o = 1.8$ kcal/mol at position 11 as compared to the wild-type operator (Table 1). The overall free energy change observed for these substitutions compared to the values obtained for the 2AP·U base pairs is 2.8 kcal/mol for position 10 and 2.6 kcal/mol for position 11.

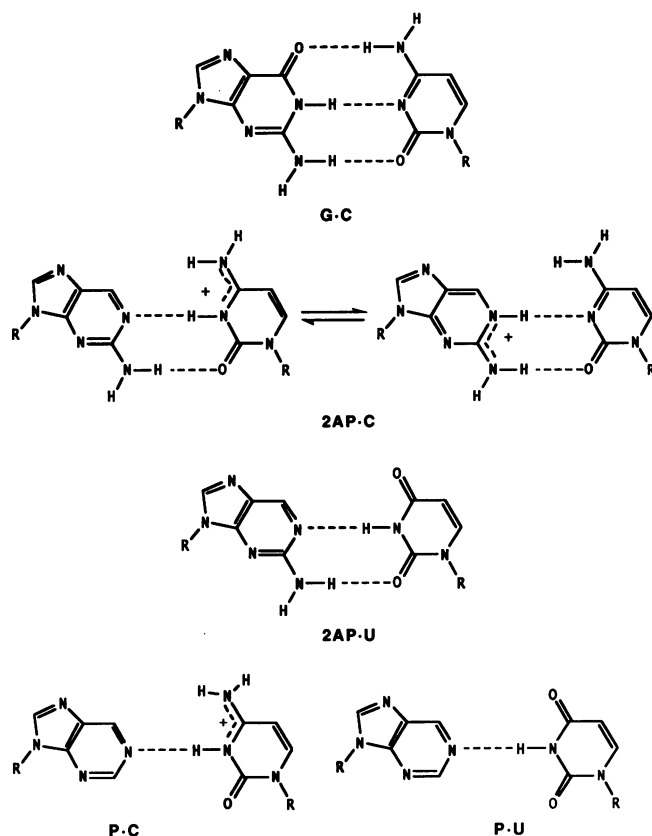


Figure 3. Proposed structures of analogue base pairs. The natural base pair G-C is shown on top. The structure of 2AP-C is shown as an equilibrium of two forms predicted by NMR and below that structure is the anomalous base pair of 2AP-U. On the bottom line the purine residue is shown paired with either cytosine or uracil. Since no detailed structural data exist for the P-C base pair, the structure depicted above is based on the 2AP-C base pair and it represents one possible pairing.

Alkylation interference of the 2AP substituted operators

In order to characterize alterations to the repressor-operator interaction, we tested the effect of the 2-aminopurine nucleoside substitutions on the chemical interference patterns of the protein-DNA complex. We have shown previously that the alkylation interference pattern of the *lac* protein-DNA complex is dependent on protein concentration (14) and have categorized the interference sites into weak, medium and strong, where weak sites are easily saturated at the lower concentrations of protein, medium sites require more protein, and strong sites never achieve saturation (Figs 5 and 6). The interference pattern of the bound and unbound DNA populations for each operator sequence was analyzed using three different concentrations of protein at a constant concentration of DNA (Figs 5 and 6). We found that only two operators showed any detectable alterations in their interference pattern and in both cases they affected adjacent phosphate groups to the modified base; these two were substitutions in positions 5 (Fig. 5B) and 10 (Fig. 6B) in the *lac* operator.

The intensity of the phosphate called p5 of the top strand in O^{2AP5} (Fig. 5B) was significantly enhanced in the bound population of the operator. At the lower concentrations of protein

where 10% of the DNA was bound, the DNA was already partitioned between bound and unbound populations (Fig. 5B, O^{2AP5} , lanes 2 and 3). This was in contrast to the wild-type operator sequence which remained the same over the protein concentrations tested and had all the labeled material located in the unbound lane.

For the position 10 substitution (O^{2AP10}), only the bottom strand was altered and this occurred at phosphate p11 (Fig. 6, O^{2AP10}). The intensities were equally partitioned between the bound and unbound populations and were unchanged with increasing concentration of protein (Fig. 6). The same phosphate site in the wild-type complex was a weak interference site and was required for the formation of the wild-type complex. We have attempted to analyze purine substituted operators and have found the breakdown of DNA at the purine site to be too great for accurate analysis.

Salt dependence of the interaction of 2AP substituted operators

To complement the alkylation interference assay, we measured the salt-dependency for the association of repressor protein to wild-type or modified operators. These experiments were performed in the standard binding buffer with varying concentrations of KCl in the range of 125–200 mM (see Fig. 7). Counterion release of the wild-type repressor-operator complex was 6.9 ± 0.4 ions. Within the experimental error of three independent measurements, the protein-DNA complexes containing the 2AP modified operators at either position 5 or 10 showed little or no net change in counterion release. The number of ions released through association of repressor with the O^{2AP10} operator was 6.5 ± 0.4 and for the complex containing a substitution at position 5 was 6.9 ± 0.5 .

DISCUSSION

To better understand the *in vitro* properties of the *lac* repressor-operator complex, we have focused on the role DNA plays in complex formation. This was achieved by inserting modified nucleotides in specific sites of the DNA operator and measuring their effect on complex formation. In our initial experiments, we made single-site modifications in the operator using two isosteric analogues of G, to systematically test the contributions of individual chemical groups to the bimolecular interaction. The first was 2-aminopurine analogue (2AP), which removes the exocyclic oxygen at position 6 of the base, and the second was the purine (P) analogue, which deletes both the exocyclic oxygen and the amino group of the base; both analogues are deprotonated at the N1 position (Fig. 2). The 2AP analogue is a major groove probe, and the P analogue alters both the major and minor grooves of the DNA. A third analogue, the nucleobase hypoxanthine (deoxyinosine), which lacks the amino group at position 2, is a probe for the minor groove and structurally complements the above analogues. The hypoxanthine analogue has already been incorporated into the *lac* operator and its effect on binding has been determined (9,34). Combined with our data that information provides valuable insight into the interaction. A direct comparison of the free energy changes between inosine and the analogues used in this work is not possible since the magnitude of binding constants is directly dependent on the ionic concentration (35,36)

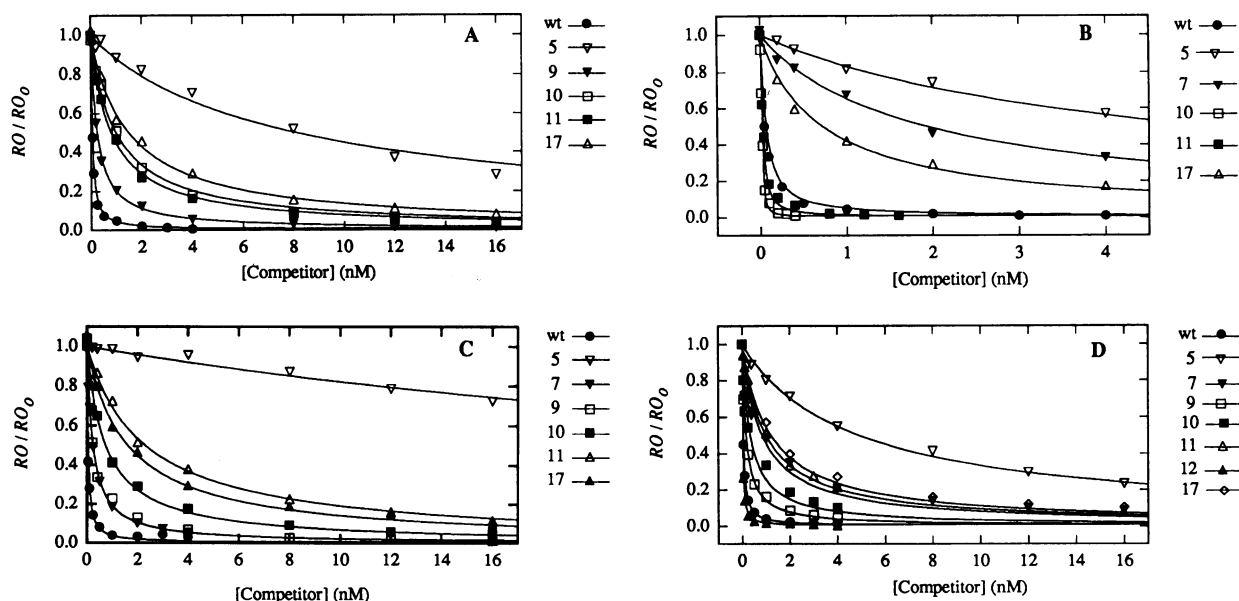


Figure 4. Measurement of the equilibrium binding constants using the competition method. Labeled wild-type operator DNA and repressor protein at a preset amount are incubated with varying concentrations of the modified operator. Each graph depicts a set of analogue substitutions used in this work as follows: (A) 2AP-C base pair; (B) 2AP-U base pair; (C) P-C base pair; (D) P-U base pair. The data points for each position tested are located next to the graph and the solid lines are the best theoretical fit of the data.

and on the length of DNA (27). Therefore, only a qualitative assessment can be made between these data sets.

From these experiments, we were able to identify a series of sites that, when replaced by modified nucleotides, destabilized the complex. Based on the available data, two categories of perturbations were apparent. The protein-DNA complex appeared to be disrupted by direct effects which resulted from positioning incompatible functional groups at the interface of the complex. The NMR data were instrumental in defining the major groove required for the interaction (16,37). The second category was evident because of the sensitivity of the complex to all substitutions made in the central region of the DNA, and we attribute this to the intrinsic DNA properties located in the central region of the operator.

Our initial observations prompted us to perform a second set of experiments that paired the modified 'purine' bases with U instead of C. Because the stability of the local DNA structure as well as surface complementarity of the two macromolecules are necessary for binding, we attempted to focus on the structural effects by altering base pairing of the DNA. By modulating the DNA duplex stability through 'anomalous' base pairs and measuring their effect on the free energy of binding, we were able to correlate the stability of the duplex with the stability of the protein-DNA complex. Previously published reports showed that the DNA duplex stability can be altered by replacing the C paired with 2AP by the T base, resulting in a higher melting temperature for the DNA (31-33,38). The reason for the increased DNA stability is apparent from NMR studies which showed that the 2AP-C forms a partially charged species to accommodate the pairing. In contrast, the pairing of 2AP-T (2AP-U base pair is shown in Fig. 3) has two well formed hydrogen bonds and therefore is more stable (31). Below we describe how these experiments, in combination with previous data, provided us with

new insights into the role of the operator in this protein-DNA interaction. One of the more interesting results was the enhancement of the stability of the complex with the insertion of anomalous base pairs in the central region of the operator.

Direct interaction of major groove sites in the operator

The insertion of the either 2AP or P analogue in the operator sequence demonstrated that the carbonyl oxygen of guanine is crucial for the wild-type interaction at either position 5 or 17, two sites that are related by a pseudosymmetric axis (Fig. 2). The effects observed for 2AP or P nucleotides base paired with uracil at these positions are entirely consistent with this interpretation (Table 1). Data obtained from NMR measurements of a fragment of the repressor (amino acids 1-56) bound to a tight-binding operator sequence (16,17,39) provide a reasonable explanation for this effect. Although it was not directly demonstrated, that Arg22 in the recognition helix of the protein interacted with base pair 5 (16,17,39). The guanidinoyl group was proposed to hydrogen bond with both the O6 and N7 atom of the guanine residue. This is in agreement with pseudorevertant genetic experiments (40,41). Recently, Chuprina *et al.* have solved the structure of the repressor headpiece bound to an 11 bp left-half operator using two-dimensional NOE measurements (16). Restrained molecular dynamics over a 85 ps time scale and annealing procedures to sample different possible structures have demonstrated that either N^H1 or N^H2 of the Arg22 sidechain interacts with the O6 of guanine, although the simultaneous participation by the N7 and O6 chemical groups is not evident.

Contrary to those experiments, we have shown that the analogue 7-deazaguanine nucleotide inserted at position 5 of the operator destabilized the complex, demonstrating that the N7 position contributes to the formation of the complex (14). The

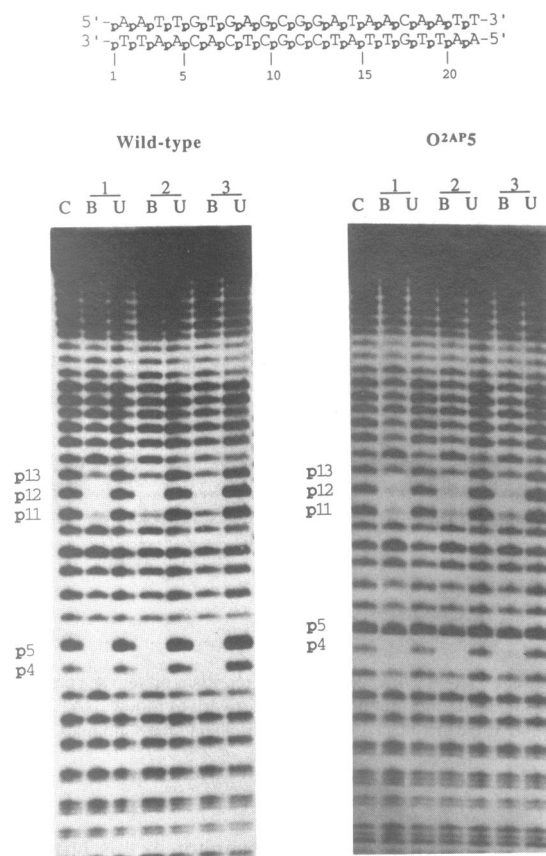


Figure 5. The phosphate alkylation interference pattern of wild-type or variant operators (Op^{2AP5}). The 21 bp of the *lac* operator wild-type sequence is shown and the phosphate sites are numbered. Symbols 'C', 'B' and 'U' represented control, bound and unbound DNA samples, respectively. On the left side is the pattern derived for the top strand of the wild-type operator. On the right side is the pattern derived for the top strand of the position 5 substitution (Op^{2AP5}). The number above the lanes refers to the following percentage bound DNA: for the wild-type: 1, 15%; 2, 30%; 3, 50%; for the top strand of the position 5 substituted operator: 1, 10%; 2, 28%; 3, 39%. The symbol 'p' represents the phosphate and the number signifies its position in the operator.

combined results are not entirely consistent with the NMR experiments and minor discrepancies are expected when comparing the intact repressor with the headpiece, even though binding by the intact repressor is mediated by interactions located in the headpiece portion of the protein (42,43).

In general, the wild-type interaction has demonstrated asymmetry mutations and substitutions in the left half of the operator always result in greater changes in affinity than those in the right half (9,14) and crosslinking experiments underscore the differences between the two halves (44). All substitutions that we have made at these two positions had a disproportionate effect on binding (Table 1). For example, the incorporation of 2AP at site 5 caused a significantly greater incremental loss in binding than the substitution at position 17. A comparison of the left-half and the right-half of the operator showed that a GTG sequence exists in the left half and not in the right half. This sequence is proposed to adopt a non-B DNA structure based on faster than anticipated proton exchange rates measured by NMR (45,46). Recent crystallographic analysis of the CAP protein bound to a DNA fragment demonstrated that this triplet exists as bent DNA (47).

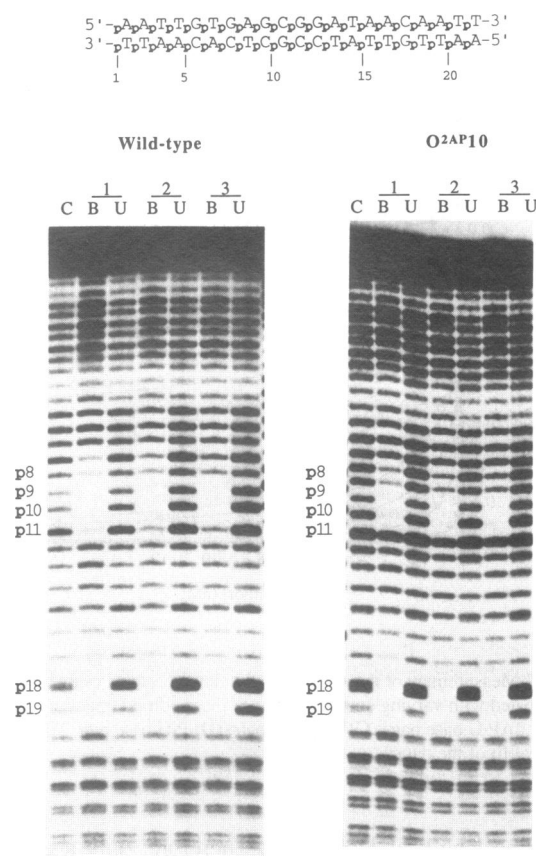


Figure 6. The phosphate alkylation interference pattern of wild-type or variant operators (Op^{2AP10}). The 21 bp of the *lac* operator wild-type sequence is shown and the phosphate sites are numbered. Symbols 'C', 'B' and 'U' represented control, bound and unbound DNA samples, respectively. On the left side is the pattern derived for the bottom strand of the wild-type operator. On the right side is the pattern derived for the bottom strand of the position 10 substitution (Op^{2AP10}). The number above the lanes refers to the following percentage bound DNA: for the wild-type: 1, 19%; 2, 44%; 3, 58%; for the top strand of the position 10 substituted operator: for the bottom strand: 1, 17%; 2, 31%; 3, 46%. The symbol 'p' represents the phosphate and the number signifies its position in the operator.

The wild-type *lac* complex may require the DNA to undergo a transition to nonB-DNA structure to maximize the interaction.

Local structural effects accompany perturbations to direct interactions

Although our data support direct interactions at these two sites, there are indications that changes in affinity are mediated by local structural considerations as well. The insertion of P instead of 2AP in the operator-protein interaction resulted in an additional loss in the observable binding constant at position 5 (Op^P5) compared with that at position 17-substituted complex (Op^P17). While this appears to support added contributions to the complex through a minor groove contact site at position 5, this is unlikely since previous work with the hypoxanthine analogue, which removes the 2-amino group from the minor groove, does not result in any significant change in the interaction (9,48).

These putative structural effects are also evident when comparing the free energy changes of 2AP-C with 2AP-U or P-C with P-U. For example, the free energy of binding for the operator

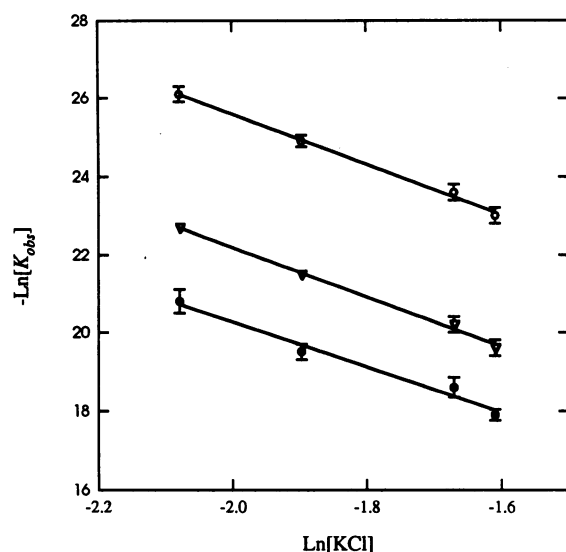


Figure 7. The effect of KCl on the observable binding constant for repressor binding to the wild-type and two singly substituted 2-aminopurine operators. The competition binding method was used to measure K_{obs} in the range of 125–200 mM of the monovalent cation. The symbols represent: wild-type operator \circ ; position 5 substitution \bullet ; and the position 10 substitution ∇ .

having the 2AP-C at position 5 is higher than 2AP-U by ~ 0.5 kcal/mol. Similar changes are evident at position 17, although not to the same extent. This analysis presumes that the substituent group (carbonyl or amino) of the pyrimidine at these positions does not perturb the interaction, an assumption consistent with available data (16,49).

Substitutions at position 7

When the purine analogue was inserted into the DNA at position 7, the free energy change was ~ 1 kcal/mol. We propose that the weaker binding complex arises from the disruption of the local helical DNA and not by the removal of a specific chemical moiety such as the minor groove amino group. Thus, singly substituted modified nucleotides at this position, such as 2AP in this work and inosine from previously published work (9), do not change the binding constant compared to wild-type, while the removal of two substituent groups with the insertion of P produces a significant effect on binding (see Table 1). The NMR data are consistent with this interpretation since headpiece bound to the operator DNA indicated that the guanine base does not participate in the protein–DNA interaction.

In contrast, the insertion of the complementary uracil at this operator position further destabilized the protein–DNA complex and is attributed to replacing the amino group with the carbonyl of U located in the major groove. These results agree with NMR data (49) that showed NOE crosspeaks between amide protons of Gln₁₈ and H5 of the cytosine at position 7 and are also consistent with ‘loss-of-contact’ genetic studies (50).

The role of the central region of the operator

The combined effects of modified nucleotides in the central base pairs at positions 9, 10 and 11 were different from substitutions

at other positions. All singly substituted operators altered the observable binding constant unfavorably, whether the modification is made in the major or minor groove. This contrasts effects associated with direct protein–DNA interactions, where some but not all chemical groups participate in complex formation, and with the position 7 substitutions where multiple groups must be removed before any effect is observed. The magnitude of the free energy of binding for either 2AP or P in the central region of the operator DNA was similar for all three sites, although the purine substitution at position 11 demonstrated a greater loss in affinity. Hypoxanthine (deoxyinosine) can also significantly disrupt the affinity of the operator–repressor complex when this analogue is positioned at these sites (9,34,48). Further, transversions (G–C) introduced into the central region resulted in a 5-fold change at position 9, a 10-fold change in binding at position 10 and a 15-fold change at position 11 (51) for a 36 base pair fragment. The combined data from the natural base and analogue substitutions demonstrated that the removal or rearrangement of any exocyclic groups always destabilize the complex. These attributes are not explainable solely by the disruption of direct hydrogen bonding schemes.

The enhanced stability of the protein–DNA complex when the anomalous base pair of 2AP-U replaced the G–C base pair in the central of the DNA provided us with valuable insight into complex formation. In two cases, the K_{obs} indicated that the complex was more stable than that measured for the wild-type operator–repressor interaction by -1.6 kcal/mol at position 10 and by -0.8 kcal/mol at position 11. Our results showed that the stability of the complex correlated with the presence of two hydrogen bonds in the central region of the operator. The response observed for repressor binding to purine–uracil substituted operators is consistent with these observations since the latter base pair, having only one possible interstrand hydrogen bond, had a more positive free energy of binding than the 2-aminopurine–uracil base pair at a given position. While it is impossible to rule out direct interactions which may be responsible for some of the loss of activity, for example through the removal of the amino group from the minor groove, the data support a significant contribution to the binding by inherent physical properties of the DNA. Interestingly, guanine paired with uracil at position 10 or 11 had free energy changes of $+0.4$ and $+0.8$ kcal/mol, respectively (X. Zhang and P. Gottlieb, unpublished results). In both cases, the amino group of guanine is positioned in the minor groove and consequently its presence is not sufficient to explain all the changes in the observable binding constant.

A closer inspection of these data indicated that simple thermodynamic stability in the central region of the operator, while important, was not a sufficient explanation for achieving complex stability. Measurements of the thermal stability of duplexes containing the various base pairs indicate that, while 2AP-U is more stable than 2AP-C, the G–C base pair is clearly the most stable of them all (18,31,32,38). Yet the G–C base pair did not produce the lowest free energy value for the protein–DNA complex. Furthermore, the stability of the natural A–T base pair is greater than the 2AP–T and three constitutive mutants have a A–T base pair in one of the three central sites. It therefore appears that while stability of the structure accounts for some of the gains in the bimolecular interaction, consideration must be given to bending, winding or kinking of the DNA, possibly in a directional manner.

Consistent with these findings are the alkylation interference and salt dependency measurements of the 2AP-U complexes.

When assessing the effect of modified nucleotides on any protein–DNA interaction, a number of changes can occur at the structural and thermodynamic level (52). From the interference studies, we found only two 2AP-substituted operators that altered the interference patterns and in both cases they occur at the phosphates adjacent to 2AP (Figs 5 and 6). There appears to be no global change in the overall phosphate pattern derived for these protein–DNA complex. These local perturbations are not accompanied by significant changes in the salt-dependent requirements for complex formation. While minor contributions to the electrostatic component of complex formation cannot be ruled out, the measured values for the modified operators tested (Fig. 7) showed little or no change in the ion release component. If the free energy change we have measured for a modified complex was entirely due to the polyelectrolyte effect (29,30), the difference in the number of ions released would be much greater. For example, based on the difference free energy of interaction measured for the wild-type and the O^{2AP10} operator, the difference between these two operator for counterion release would be ~ 1.85 . From these measurements, we propose that most of the changes in affinity are associated with perturbations to the non-electrostatic component of the interaction. The use of the new base pair allowed the reconstitution of the alkylation interference pattern to that of the wild-type pattern (data not shown) and we assume that salt dependent component of the association process remains unaffected.

We have also analyzed operator DNA which contained a double substitution of the 2AP·U base pairs at positions 10 and 11 and found that the observable binding constant for this operator sequence was -0.6 kcal/mol (data not shown). The combined energies of each of the singly substituted operators is -2.4 kcal/mol, indicating that energies associated with the central region of the DNA are non-additive. This is expected for adjacent base pair sites involved in the association process and that influence the affinity of the complex through the inherent properties of the DNA.

An important insight into the role of the central region of the operator can be found in the recently solved structure of the PurR repressor bound to its DNA site published recently (6). This structure is representative of the class of DNA binding proteins of which the *lac* repressor is a member. Brennan and co-workers have noted that two helices (helix 4) of the protein are positioned in the minor groove of the central region of the DNA binding site, resulting in the insertion of two leucine residues between the bases. The DNA in the complex has a kink of $\sim 45^\circ$ centered around the minor groove insertion site. Because the *lac* repressor is considered homologous to the PurR protein in the DNA binding region, the kink evident in the PurR complex is thought to exist in the *lac* repressor–operator complex as well. Our results with the analogue base pairs are in agreement with this predication.

Enhancement of the stability of the complex in the presence of the anomalous base pairs reflects specific properties of the DNA that facilitate the required transition in the complex. The modified base pair in the central region of the protein–DNA complex influences the association process by altering the intrinsic properties of the operator DNA, lowering the energetic barrier associated with the transition of free DNA to its bound form. The exact mechanism required to achieve complex specificity and stability will require a more detailed analysis of the physical properties of the DNA. The 2AP·U base pair is unusual in that it has two hydrogen bonds like an AT base pair, but unlike the AT

base pair, a hydrogen bond is located in the minor groove and not in the major groove. This structure has no real counterpart in natural DNA and contributes to the interaction in yet unknown ways. Perhaps the 2AP·U base pair allows the major groove to be compressed more easily and as a result stabilizes the protein–DNA complex. This putative ability of the 2AP·U base pair to lower the transitional roll into the major groove may be useful for diagnosing these effects in other protein–DNA complexes.

Intrinsic properties of the DNA in solution have been correlated with functional activity in a number of systems. For example, the recent work of Koudelka and Carlson showed that the intrinsic flexibility and twisting in the central base pair region of the 434 repressor binding site plays a key role in creating high or low affinity binding (53); the greater the flexibility, the lower the transition energy required to convert unbound DNA to an alternative structure that produces a more stable complex. Recent experiments with the *trp* repressor–operator interaction and various modified nucleotides revealed that this complex may undergo significant structural changes during the formation of the complex as well (7). The *lac* repressor appears to stand in contrast to the 434 repressor in its apparent binding requirements for the central region. The affinity of 434 repressor–operator is weakened with the guanine residues positioned in the central core while the *lac* repressor–operator interaction maintains a high affinity complex with these bases. Conversely, the presence of a A·T residue in this region of the *lac* operator tends to weaken the affinity, as was shown for three *lac* repressor constitutive mutants.

Recent work in the *EcoRI* system has dissected contributions of the P·U base pair at a specific positions in the restriction site. A single P·T nucleotide substituted position served to enhance the stability of the protein–DNA interaction, presumably by facilitating the transition of the DNA structure to the kinked form (54). In that case compensating effects are observed which in sum produces a net favorable change on the binding interaction. We have observed a similar improvement in binding at position 12. Because our system does not have enzymatic activity and because no structure is available which reports on the details of the DNA, we are unable to exclude such compensatory changes in our work. However, this does not alter the general conclusions that we have made about indirect effects on binding.

Our results underscore the essential role that DNA sequence plays in the association of the repressor to operator. While the crystallographic data of the PurR have been used to argue that two leucines actively open the DNA, the mechanism of binding will no doubt depend on the intrinsic ability of the DNA to be opened. In light of our results for the central region of the operator and assuming that PurR and LacI have a common recognition element, the operator DNA appears to have intrinsic properties that contribute significantly to binding and sequence discrimination by the protein.

Summary

The use of modified nucleobases inserted into DNA recognition sites is a powerful tool that allows new insights into protein–DNA interactions. The work we have presented has centered around the use of analogue bases that are substituted at single sites or as complementary base pairs, allowing us to dissect direct and indirect influences on the affinity of the *lac* protein–DNA complex. Interpretation of our results is made possible because of information available from previous thermodynamic and struc-

tural studies which analyzed how these inserted nucleobases affect the properties of DNA duplexes. More information will be gained about protein-DNA interactions, as we continue to expand the repertoire of DNA analogues and analyze how they influence macroscopic properties of DNA.

For this protein-DNA complex, we have presented evidence that supports hydrogen bonding contact sites at positions that are predicted to be directly involved in the formation of the protein-DNA interface. We have also demonstrated that the central region of the operator is responsible, in part, for modulating the protein-DNA complex indirectly and by manipulating these indirect effects with 'anomalous' base pairs a protein-DNA complex more stable than the wild-type operator-repressor was achieved.

ACKNOWLEDGEMENTS

We thank Mitch Lewis, Helen Pace and Ponzy Lu for the gift of *lac* repressor and Helen Pace for comments on this manuscript.

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